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It has been shown that dietary n-6 polyunsaturated fatty acids (PUFAs) promote tumorigenesis and maintain growth of breast cancer cell lines, while n-3 PUFAs generally reduce the incidence and development of cancer and inhibit growth in cell culture. We investigated the effects of various n-6 and n-3 PUFAs, on lipogenic gene expression in two breast cancer cell lines, MDA-MB-231 a p53-/- cell line and MCF-7 a p53+/+ cell line. In both lines, we showed that PUFAs repressed the expression of two important genes in the fatty acid biosynthesis pathway, fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1). In addition we showed that this PUFA-induced repression was mediated through the sterol regulatory element binding protein 1a (SREBP-1a) and not the mutant p53 protein as we had previously hypothesized. SREBP is a newly identified and critical central activator of both the cholesterol and fatty acid biosynthesis pathways. Two SREBPs isoforms (SREBP-1 and 2) are expressed in normal tissues. We found that the breast cancer cells only express the SREBP-1a isoform at very high levels consistent with the ability of these cells to carry out high levels of de novo fatty acid synthesis for membrane biogenesis .

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(5) INTRODUCTION

An effective treatment for cancer has remained elusive for decades as various therapeutic approaches have yielded mixed results. One therapeutic strategy that has been long overlooked, however, involves exploiting a striking difference in cellular fat metabolism observed between normal and cancer cells. Normal cells in most body tissues synthesize only low amounts of fatty acids; if fatty acids are needed by cells for functions such as cell membrane composition or energy storage, cells will preferentially utilize fatty acids obtained exogenously from dietary sources (1-5). On the other hand, many types of cancer cells synthesize high amounts of fatty acids, even in the presence of sufficient quantities of dietary fatty acids. Elevated synthesis of fatty acids is presumably necessary to accomodate the accelerated growth observed in cancer cell lines and the concomitant increased requirement for fatty acids in cell membrane biosynthesis (6).

In this study, we investigated the effects of various n-6 and n-3 polyunsaturated fatty acids (PUFAs), CLA isomers, and cholesterol on lipogenic gene expression in two breast cancer cell lines, MDA-MB-231 and MCF-7. The MDA-MB-231 line harbors a mutation in both alleles of its p53 gene, a tumor suppressor gene mutated in about 50% of all human cancers (7). When this cell-cycle regulatory gene is mutated, cell division occurs uncontrollably and is unregulated. Conversely, the MCF-7 breast cancer cell line possesses a wildtype p53 gene. Consistent with their p53 status, it is observed that the MDA-MB-231 cell line grows and divides at a much higher rate than the MCF-7 line and other non-cancerous cell lines. Originally we hypothesized that PUFAs inhibit cell proliferation associated with fatty acid synthesis by regulating the interaction of the mutant p53 protein with the specific motif on the promoters of the genes involved in fatty acid biosynthesis. Instead, we have found that another transcription factor termed sterol regulatory element binding protein (SREBP) mediates PUFA repression of the SCD-1 gene in breast cancer cells

(6) BODY

(i) Materials and Methods

Cell culture and lipid treatments - MDA-MB-231 and MCF-7 breast cancer cell lines were cultured at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine. At 80-90% confluency, the cells were washed with PBS twice and then refed with Williams medium E (4.5 g/mL glucose) containing 1% penicillin/streptomycin and various PUFAs (100-300 μM) and cholesterol. All PUFAs were complexed to bovine serum albumin, and cholesterol compounds were dissolved in 100% ethanol. The treated cells were then incubated at 37°C and 5% CO_2 for 24 hours, and harvested thereafter to obtain total RNA or protein extracts.

Northern blots - Following lipid treatments, total cellular RNA was harvested and isolated according to the guanidine thiocyanate lysis method (8). Formaldehype-agarose gels were run with 20 μg of total RNA for each sample, and then RNA was transferred by capillary action to Nytran membranes. Blots were hybridized in Images_{TM} buffer with cDNA probes for fatty acid synthase, a 1.5-kb fragment of human stearoyl-CoA desaturase, and pAL15 marker. Probes were labeled by random priming using [α^{32}-P]deoxycytidine 5′-triphosphate and 100 ng of the DNA fragment.

Western blots - Following treatments with lipids, total cellular protein was harvested using the RIPA lysis method. Protein samples (25-40 μg of protein) and Bio-rad molecular weight standards were electrophoresed on a denaturing 10% SDS-polyacrylamide gel, and then transferred to immunobulin membranes via electroblotting. Western blotting was performed by blocking for 2 hours in 10% milk in either TBS-T or PBS-T, followed by incubations with primary (2 hrs.) and secondary (1 hr.) antibodies in 1-5% milk in TBS-T or PBS-T. A monoclonal antibody against SCD-1 and peroxidase-labeled anti-rabbit IgG were used to detect SCD protein expression. All blots were exposed via the ECL detection method.

Transient DNA transfections and CAT assays - For transient transfection of MDA-MB-231 cells were split onto 6-cm dishes in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were allowed to grow 12-24 hours at 37°C and 5% CO_2 to 80-100% confluence. Cells were then co-transfected via the calcium phosphate co-precipitation method with 10 μg of SCD promoter/CAT reporter constructs (pCAT-An vector) and 20-200 ng of a plasmid containing the mature form of SREBP-1a (pPac vector). After 16-24 hours of incubation, cells were washed with PBS and then treated in Williams medium E supplemented with PUFAs and/or cholesterol. Following 24 hours of treatment, the cells were harvested for the CAT enzyme assay. CAT activity, which is measured as the amount of [^{3}H]acetylchloramphenicol formed over time, is a quantitative measure of SCD promoter activity.

(ii) Results

Elevation of fatty acid synthase mRNA expression in MDA-MB-231 cells. - To examine the effects of PUFAs and cholesterol on the expression of fatty acid synthase in the breast cancer cell lines, MDA-MB-231, MCF-7, and HepG2 cells (a hepatic cell line in which the PUFA and cholesterol-regulated expression of FAS and SCD is known) were treated with 300 μM arachidonic acid (AA, 20:4n-6) and cholesterol (10 μg/mL) supplemented with 25-hydroxy-cholesterol (1 μg/mL). Northern blot analysis showed that MDA-MB-231 cells expressed much higher levels of FAS messenger RNA than either the MCF-7 or HepG2 cells, regardless of treatment, and MCF-7 levels were considerably lower than the HepG2 levels (Fig. 1). Relative to the albumin control, arachidonic acid had no significant effect on FAS expression in MDA-MB-231 cells but repressed in the MCF-7 and HepG2 cells. Cholesterol repressed FAS expression in the MDA-MB-231, MCF-7 and HepG2 lines. Cholesterol is known to effect repression of fatty acid synthase in the HepG2 cell line (9).

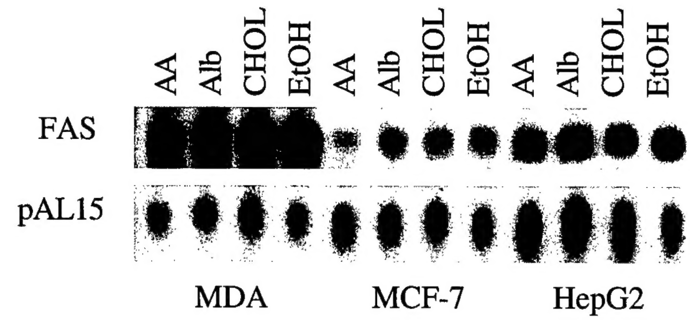

Figure 1. **Fatty acid synthase is expressed at higher levels in the MDA-MB-231 breast cancer cell line.** MDA-MB-231 and MCF-7 breast cancer cells as well as HepG2 hepatic cells were treated for 24 hrs. with 300 μM arachidonic acid (AA) and its control (Albumin), and cholesterol (10 μg/mL cholesterol plus 1 μg/mL 25-OH cholesterol) and its control (100% ethanol). 20 μg of total RNA were electrophoresed on a 1% formaldehyde agarose gel and transferred to a Nytran membrane. The northern blot was hybridized with a 32-P-labeled probe for fatty acid synthase and pAL15 (a control for equal loading of RNA).

SCD mRNA expression in MDA-MB-231 and MCF-7 cell lines - To examine the effects of n-6 PUFAs, CLAs, and cholesterol on the expression of SCD in the breast cancer cell lines, MDA-MB-231 and MCF-7 cells were treated with arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), linoleic acid (LA, 18:2n-6), cholesterol (10 μg/mL) supplemented with 25-hydroxy-cholesterol (1 μg/mL), *cis*9,*trans*11-CLA, *trans*10,*cis*12-CLA, and a mixture of naturally-occurring forms of CLA. All concentrations of the PUFAs were 100 μM, except for the isolated CLA isomers at 50 μM.

From Northern blot analysis, both of the breast cancer cell lines expressed very low levels of SCD mRNA relative to day 6 3T3-L1 mouse adipocytes. The standard n-6 PUFAs, AA and LA, both repressed SCD gene expression in each of the cancer lines (Fig. 2A). In addition, cholesterol decreased the level of SCD mRNA in MCF-7 cells, but there was no significant change (relative to Albumin control) in SCD expression upon cholesterol treatment in the MDA-MB-231 cell line (Fig. 2A).

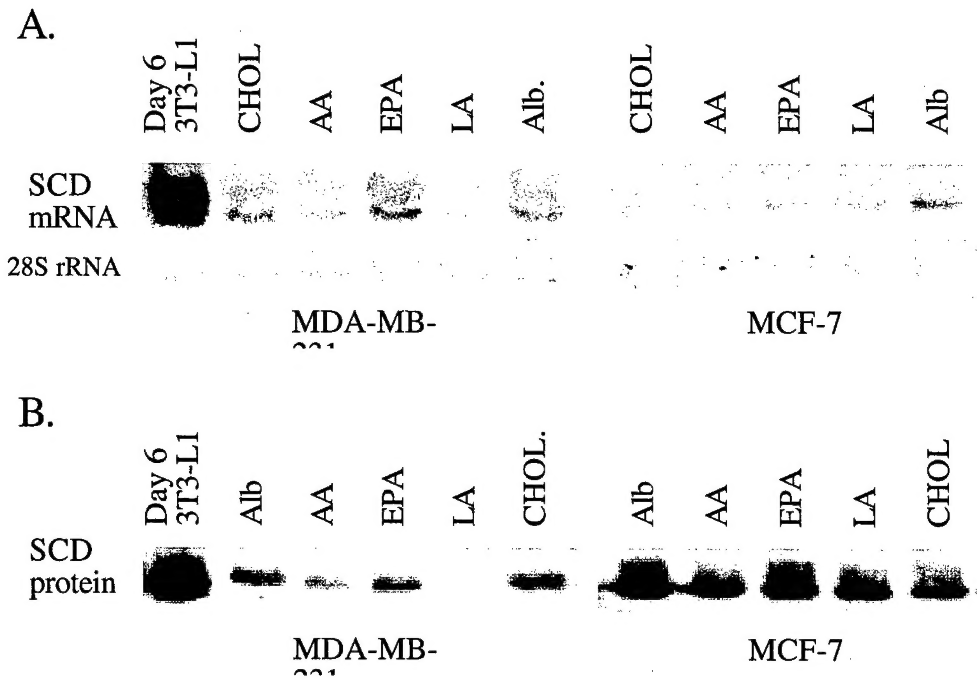

Figure 2. **Effects of PUFA and cholesterol treated on the expression of stearoyl-CoA desaturase mRNA and protein in the MDA-MB-231 and MCF-7 breast cancer cell lines.** MDA-MB-231 and MCF-7 breast cancer cells were each treated for 24 hrs. with 100 µM concentrations of arachidonic acid (AA), eicosapentaenoic acid (EPA), linoleic acid (LA), and cholesterol (10 µg/mL cholesterol plus 1 µg/mL 25-OH cholesterol). Both lines were also treated with bovine serum albumin (Alb.) as a control, which was complexed to all PUFAs. RNA and protein from day 6 3T3-L1 mouse adipocytes was also added as a control for SCD expression. **A.** 20 µg of total RNA were electrophoresed on a 1% formaldehyde agarose gel and transferred to a Nytran membrane. The northern blot was hybridized with a 32-P-labeled probe for stearoyl-CoA desaturase. Equal loading of total RNA is demonstrated through levels of 28 rRNA. **B.** 40 µg of total cellular protein were run on SDS-PAGE, and transferred to nitrocellulose membrane. Western blots were probed with a monoclonal SCD antibody and horseradish peroxidase anti-rabbit IgG antibody.

The CLAs yielded dissimilar effects of SCD expression. In the MDA-MB-231 cells, the c9,t11-CLA isomer slightly elevated SCD expression, while the t10,c12-CLA isomer and the CLA mixture both significantly reduced SCD levels. In MCF-7 cells, c9,t11-CLA slightly reduced SCD mRNA levels, but both t10,c12-CLA and CLA elevated SCD expression (Fig. 3A).

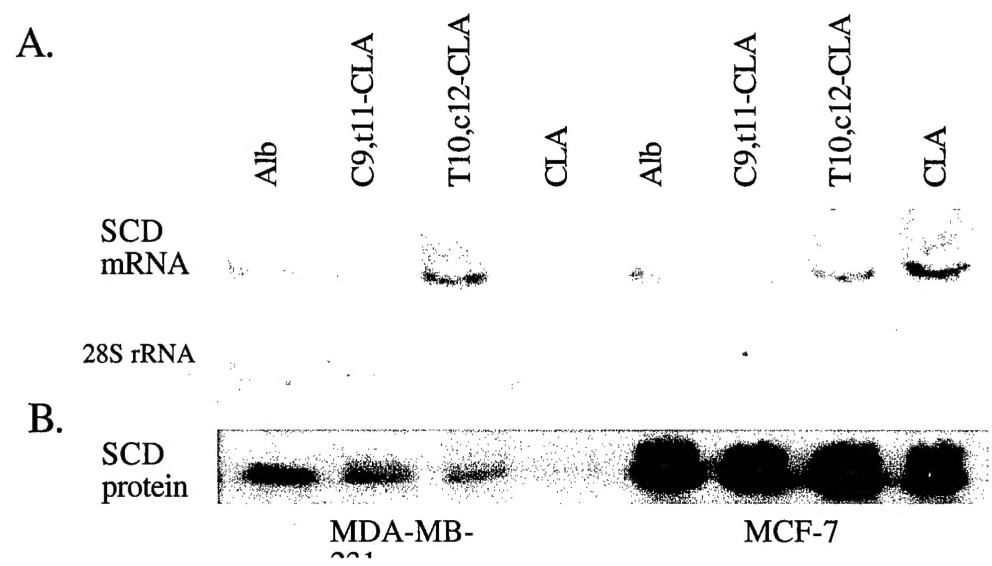

Figure 3. **Effects of conjugated linoleic acid (CLA) treatments on the expression of stearoyl-CoA desaturase mRNA and protein in the MDA-MB-231 and MCF-7 breast cancer cell lines.** MDA-MB-231 and MCF-7 breast cancer cells were each treated for 24 hrs. with 50 μM c9,t11-CLA, 50 μM t10,c12-CLA, and 100 μM CLA. Both lines were also treated with bovine serum albumin (Alb.) as a control, which was complexed to all CLAs. RNA and protein from day 6 3T3-L1 mouse adipocytes was also added as a control for SCD expression (as shown in Fig. 2). **A.** 20 μg of total RNA were electrophoresed on a 1% formaldehyde agarose gel and transferred to a Nytran membrane. The northern blot was hybridized with a 32-P-labeled probe for stearoyl-CoA desaturase. Equal loading of total RNA is demonstrated through levels of 28 rRNA. **B.** 40 μg of total cellular protein were run on SDS-PAGE, and transferred to nitrocellulose membrane. Western blots were probed with a monoclonal SCD antibody and horseradish peroxidase anti-rabbit IgG antibody.

SCD protein expression in MDA-MB-231 and MCF-7 cell lines - The levels of SCD protein upon treatment with the same PUFAs, cholesterol, and CLAs as mentioned for the SCD northern blot analysis were examined via western blotting. The expression of SCD protein was much higher in the MCF-7 cell line as opposed to MDA-MB-231. In correspondence with the SCD northern blot, the n-6 PUFAs (AA and LA) repressed SCD protein levels in both cell lines. Cholesterol down-regulated SCD in MCF-7 lines, while having no significant effect in MDA-MB-231 (Fig. 2B). The CLAs all dramatically increased SCD protein in the MCF-7 extracts; in the MDA-MB-231 line, all CLAs repressed SCD, with CLA repressing over 50% (Fig. 3B).

SREBP-1 and SREBP-2 protein expression in MDA-MB-231 and MCF-7 cell lines - To determine (1) if PUFAs exhibited an effect on SREBP protein expression in the breast cancer cell lines and (2) whether levels of mature SREBP protein correlated with the observed effect of PUFAs on SCD, western blot analysis using SREBP-1a and SREBP-2 antibodies was performed. MDA-MB-231, MCF-7, and 25-RA (a chinese hamster ovary cell line in which production of the mature form of SREBP is constitutively activated) cells were treated with arachidonic acid (AA), linoleic acid (LA), cholesterol, and and bovine serum albumin as a control. In the MDA-MB-231 cell line, both PUFAs and cholesterol slightly repressed SREBP-1a protein expression relative to the albumin control, while the PUFAs and cholesterol induced greater repression of SREBP-1a in the MCF-7 cells (Fig. 4). The SREBP-2 was undetectable by western blotting in all treatments of the breast cancer cell lines. Both SREBP genes were expressed in the 25-RA cell line.

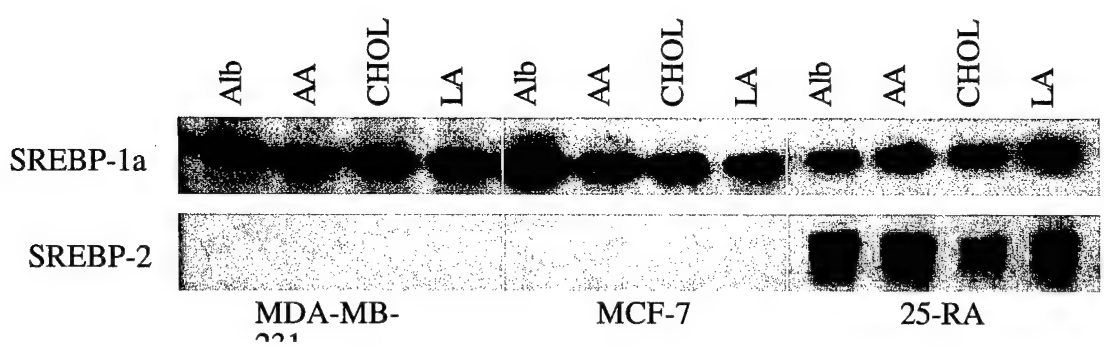

Figure 4. **Effects of PUFA and cholesterol treatments on the expression of sterol regulatory element binding proteins 1a and 2 (SREBP-1, SREBP-2) in the MDA-MB-231 and MCF-7 breast cancer cell lines and the 25-RA cell line.** MDA-MB-231, MCF-7, and 25-RA cells were each treated for 24 hrs. with 200 μM concentrations of arachidonic acid (AA) and linoleic acid (LA), cholesterol (10 μg/mL cholesterol plus 1 μg/mL 25-OH cholesterol), and albumin as a control. 50 μg of total cellular protein were run on SDS-PAGE, and transferred to nitrocellulose membrane. Western blots were probed either with the monoclonal anti-SREBP-1a (CRL 2121) or SREBP-2 (CRL 2198) antibody, and the secondary peroxidase anti-mouse IgG antibody.

Regulation of SCD-1 and SCD-2 promoters by SREBP and PUFA in transiently transfected HepG2 cells. - To observe the regulatory effects of mature SREBP-1a and PUFAs on the activity of SCD promoters, HepG2 hepatic cells were transiently co-transfected with 0, 50, or 200 ng (per 6-cm dish) of plasmid DNA containing the sequence encoding mature SREBP-1a and either 600-SCD1/CAT or 580-SCD2/CAT promoter/reporter constructs. After transfection, the cells were then refed with cholesterol or both arachidonic acid and cholesterol. Relative to no lipid treatment, cholesterol repressed the SCD promoter activities over 50% (Fig. 5). Cell transfected with mature SREBP-1a increased 600-SCD1 and 580-SCD2 promoter activities by 2-fold and 3- to 5-fold, respectively (relative to cholesterol treatment only and no SREBP transfected). In addition, arachidonic acid treatment repressed SREBP-induced activation of 600-SCD1 and 580-SCD2 promoter activities by over 50% and by 50-90%, respectively.

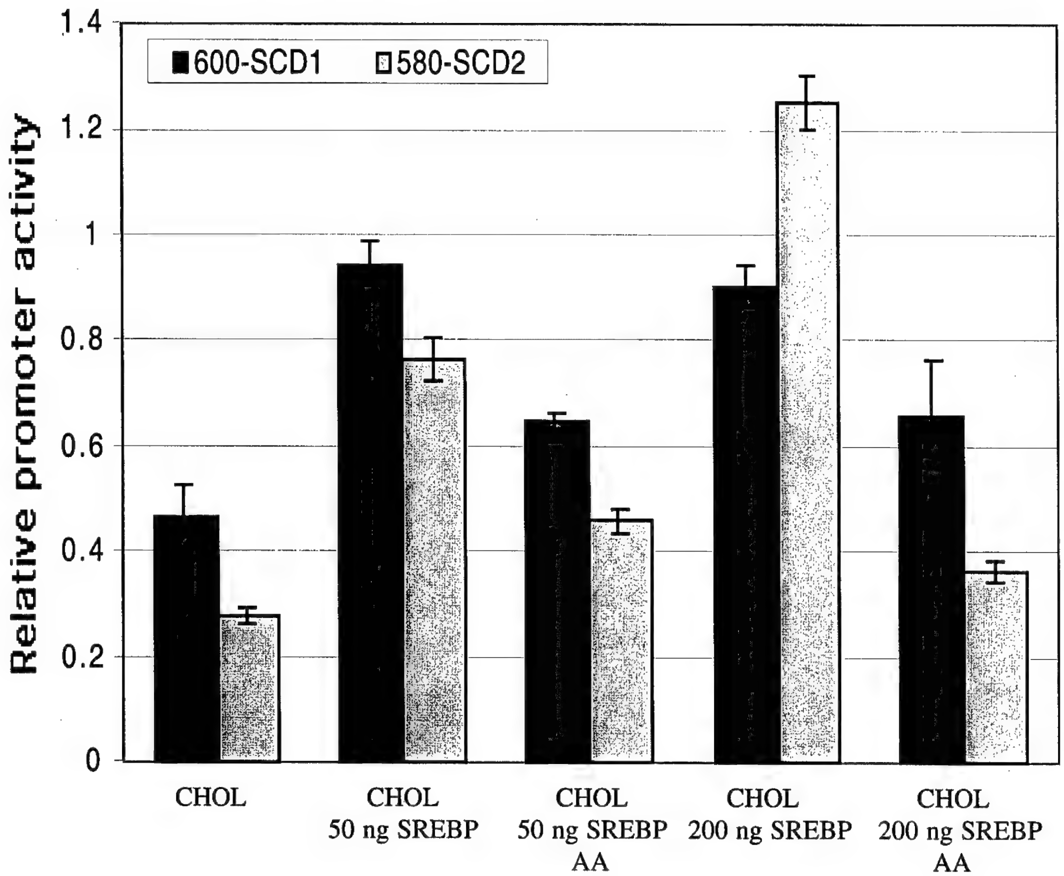

Figure 5. **Transient co-transfection of SCD-1 and SCD-2 promoters with mature SREBP-1a in HepG2 cells.** HepG2 cells were transiently co-transfected either with 10 μg of 600-SCD1/CAT or 580-SCD2/CAT promoter/reporter plasmid and varying amounts (0, 50, or 200 ng) of mature SREBP-1a in a pPac expression vector. Cells were incubated for 16-24 hours, and then refed with cholesterol, both 200 μM arachidonic acid and cholesterol, or no treatment. After 24 hours of treatment, cells were harvested for CAT assays. The promoter activities are relative to the respective 600-SCD1 and 580-SCD2 CAT activities without cholesterol or PUFA treatment (i.e., 1.0).

(iii) Discussion

Expression of fatty acid synthase in breast cancer cell lines. - The elevated level of fatty acid synthase mRNA expression in MDA-MB-231 cells relative to normal HepG2 hepatic cells was consistent with observations of high FAS levels in carcinomas (4, 5). This was also compatible with the accelerated growth rate of these cells; it is conjectured that faster-growing, aggressive cells require increased amounts of fatty acids for membrane biosynthesis and energy storage. FAS is a critical and central enzyme in the fatty acid biosynthesis pathway because it catalyzes the formation of saturated fatty acids, which are also precursors for many monounsaturated and polyunsaturated fatty acids. The MCF-7 cells, on the other hand, expressed significantly low levels of FAS. This observation was consistent with their slower rate of growth, but contrary to the majority of research findings which reveal high FAS expression in most breast carcinomas and cell lines (4, 5).

In general, these results were in agreement with the hypothesis that cancer cell lines possessing mutations in the p53 tumor suppressor gene correspondingly exhibit high levels of fatty acid synthesis. Indeed, this was reflected in the levels of FAS expressed by MDA-MB-231 and MCF-7 harboring mutant and wildtype p53 genes, respectively. To identify a correlation between p53 status and lipogenic gene expression, however, additional cancer cell lines must be included in this experiment.

Levels of SCD expression in breast cancer cell lines - From northern and western blot analyses, MDA-MB-231 cells expressed low levels of stearoyl-CoA desaturase mRNA and protein. This result was unexpected because FAS, which produces a preferential substrate for SCD (palmitic acid), was highly expressed in this cell line; in addition, the products of SCD are the major constituents of cell membranes (10). This result was also contrary to the finding that SCD is expressed at high levels in cultured hepatoma cells (11). Nevertheless, these observed levels of SCD mRNA and protein may be sufficient for high

production of SCD fatty acid products. In this cell line, SCD may be regulated under post-translational control mechanisms; SCD may have a low rate of protein turnover, allowing for increased amounts of catalysis per individual enzyme.

The MCF-7 cancer cell line expressed low levels of SCD mRNA, consistent with FAS expression in these cells. However, relative to MDA-MB-231, these cells expressed much higher levels of the SCD protein, comparable to day 6 3T3-L1 adipocytes. It is possible that SCD is regulated by post-transcriptional mechanisms in this cell line. For instance, SCD protein levels could increase as a result of greater mRNA stability or faster rates of translation, in effect elevating the amount of SCD protein produced. It is important, nevertheless, to realize that the levels of SCD protein may not necessarily reflect high rates of catalysis – SCD in MCF-7 cells may be regulated by a high protein turnover rate.

Role of SREBP-1a in PUFA-induced repression of SCD in cancer cells. - SREBP-1a, a transcriptional activator of many genes in the fatty acid biosynthesis pathway (12), is speculated to mediate PUFA-induced repression of SCD in normal, non-cancerous cells in liver and adipose tissue. In the MDA-MB-231 breast cancer cell line, PUFAs (arachidonic acid and linoleic acid of the n-6 series) exhibited no effect on the expression of mature SREBP-1a, but generally decreased SCD expression. This provided evidence that the PUFA-mediated repression of SCD was SREBP-independent in this cell line; PUFAs may induce SCD repression via other transcription factors or pathways. In addition, cholesterol did not affect levels of SREBP-1a or SCD expression. In many normal liver and adipose tissues, cholesterol usually inhibits the maturation of SREBP into its mature, active form as a transcription factor. Furthermore, this cell line did not express detectable levels of SREBP-2, a major activator of the cholesterol biosynthesis pathway. Thus, this cell line may also have a defect in the regulation of cholesterol biosynthesis.

In the MCF-7 breast cancer cell line, the n-6 PUFAs repressed both SREBP-1a and SCD expression. This observation is consistent with an SREBP-mediated mechanism of

PUFA-induced repression of SCD; that is, it is in agreement with the hypothesis that PUFAs reduce SREBP-1a expression, which in turn transcriptionally activates decreased levels of SCD. Unlike the MDA-MB-231 cell line, cholesterol significantly repressed both SREBP-1a and SCD expression in MCF-7 cells. It is reasonable to speculate that SCD may be down-regulated by the effect of cholesterol in suppressing SREBP-1a maturation (as is observed in normal cells). Similar to MDA cells, however, SREBP-2 was not expressed at detectable levels in MCF-7; thus, this cell line may also exhibit a defect in the regulation of cholesterol biosynthesis. Altogether, the differences between the two cancer cell lines in the effects of PUFAs on SREBP-1a expression and by cholesterol on SCD regulation as well as the lack of SREBP-2 expression in both lines may be critical to their mechanisms of fatty acid biosynthesis regulation and growth.

The effect of the conjugated linoleic acids on SCD expression was also noteworthy. CLAs, which have been shown to exhibit an anticarcinogenic effect, induced dissimilar effects on SCD expression between the two breast cancer cell lines. Although the northern blot analysis yielded a few inconclusive results, western blots showed that the CLAs generally repressed SCD expression in MDA-MB-231 cells, while no effect was observed on the SCD protein levels in the MCF-7 cell line. The mechanism through which these effects occur is yet to be investigated.

Evidence of SREBP interaction with SCD promoters and mediation of PUFA-induced repression of SCD through mature SREBP - The transient co-transfection of MDA cells revealed an interaction between the mature SREBP-1a protein and the SCD-1 and SCD-2 promoters. With levels of endogenous SREBP reduced by cholesterol treatment, SREBP activated both the 600-SCD1 and 580-SCD2 promoters by 2 to 5-fold. In previous transient co-transfection experiments in Drosophila insect cells containing no endogenous SREBP, mature SREBP-1a induced 600-SCD1 promoter activity by 6-fold (data not shown). The magnitude of activation is considerably lower than that demonstrated

with other lipogenic gene promoters, such as fatty acid synthase and acetyl-CoA carboxylase. However, these studies have also shown that optimal activation through SREBP usually occurs with the presence of a co-activating transcription factor. Transient co-transfection experiments on other lipogenic gene promoters have identified Sp1, nuclear factor-Y, and other ubiquitous transcription factors as co-activators with SREBP , but a co-activator has not been uncovered for SCD promoter interactions. We are checking to determine whether this co-activator protein is p53.

(7) KEY RESEARCH ACCOMPLISHMENTS:

1. PUFAs repressed the expression of two important genes in the fatty acid biosynthesis pathway, fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1).

2. In addition we showed that in the MCF-7 cells PUFA-induced repression was mediated through the sterol regulatory element binding protein 1a (SREBP-1a)

3. SREBP is a newly identified and critical central activator of both the cholesterol and fatty acid biosynthesis pathways. Two SREBPs isoforms (SREBP-1 and 2) are expressed in normal tissues. We found that the breast cancer cells only express the SREBP-1a isoform at very high levels consistent with the ability of these cells to carry out high levels of de novo fatty acid synthesis for membrane biogenesis .

(8) REPORTABLE OUTCOMES

- Abstract to the FASEB meetings in San Diego CA, April 2000

(9) CONCLUSIONS

In the current study using the two breast cancer cell lines both the n-3 and n-6 polyunsaturated fatty acids repressed lipogenic gene expression. Future studies will address the differential effects of the n-3 versus the n-6 fatty acids on the progression of breast cancer. We noted though, in all cases the n-3 fatty acids were more potent than the n-6 in repressing gene expression. Whether this is related to the statement that n-3 polyunsaturated fatty acids are more potent in preventing breast cancer is not clear at the present time.

The involvement of the SREBP transcription factor is very interesting because it links the regulation of genes of fatty acid biosynthesis to those of cholesterol metabolism. Independent of the mechanism of PUFA action on SREBP or p53 protein, the gene aspect of this study could be used as a more accurate means of screening for the potency of polyunsaturated fatty acids towards the growth of cancer cells.

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(11) APPENDICES

362.5

Differential Control of Stearoyl CoA Desaturase Gene Transcription by n-3 and n-6 Polyunsaturated Fatty Acids

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Polyunsaturated fatty acids (PUFAs) play important roles in the control of cellular differentiation as well as in the regulation of transcription of genes encoding enzymes of carbohydrate and lipid metabolism. It has been established that one of the primary mechanisms for the control of lipogenic genes is the maturation of the sterol regulatory element binding protein (SREBP). Upon nuclear localization, mature SREBP is an inducer of transcription. It is established that levels of mature SREBP levels will decrease with treatments of PUFAs and cholesterol thereby decreasing the transcription of target genes, but by using Chinese hamster ovary (CHO) cell lines with spontaneous mutations in SREBP maturation pathways, we have seen a decrease in expression of SCD in the absence of changing SREBP levels. Furthermore, n-3 PUFAs appear to be more potent than n-6 PUFAs in repressing SCD gene expression. Analysis of mRNA, promoter activity of SCD1, and protein levels in these cells indicate the existence of a SREBP-independent mechanism of transcriptional regulation. It is believed that a putative PUFA binding protein exists and has the ability to monitor the PUFA content of the cell and control transcription. In addition, this protein or family of proteins can distinguish between the n-6 and n-3 families of PUFAs.

362.6

Cloning of two alternately spliced transcripts of bovine hepatic pyruvate carboxylase

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Pyruvate carboxylase (PC; EC 6.4.1.1) is important for gluconeogenesis, fatty acid synthesis, and amino acid catabolism. The role of PC in bovine liver is particularly critical because ruminal fermentation limits availability of dietary glucose for absorption, therefore the animal relies heavily on the hepatic gluconeogenesis. Primers corresponding to regions of identity between human, rat, and mouse PC were used to clone a 420 bp segment of bovine PC (bPC1) by RT-PCR. The bPC1 clone contains 90% identity to the corresponding coding region of human PC. Northern blot analysis indicates that bPC1 binds to a single 4200 base transcript of bovine RNA which is similar in size to human PC mRNA. The 5' untranslated region(s) (UTR) of bovine PC were cloned using 5' rapid amplification of cDNA ends and three separate gene specific primers designed from bPC1. Two clones were identified containing a 70 (bPC5'1) and 255 (bPC5'2) bp 5' UTR linked to the coding sequence for bovine PC. The 70 bases of bPC5'1 and the first 70 bases of bPC5'2 are identical which suggests they originate from a single promoter via alternate splicing. The sequence identity of bPC5'1 and bPC5'2 with two forms of human and five forms of rat 5' UTRs range between 25 and 47%. Primer extension analysis confirms the presence of the bPC5'1 transcript and indicates three additional PC transcripts for bovine liver. Identification of the different 5' UTRs is a first step in understanding the regulation of bovine PC. Data points to the possibility of regulation for bovine PC that is similar to regulation of PC transcripts for species where multiple 5' UTRs have been identified.

362.7

EFFECT OF LONG-CHAIN UNSATURATED FATTY ACIDS ON PORCINE ADIPOCYTE DIFFERENTIATION AND ON TRANSCRIPTS FOR ADIPOCYTE-RELATED GENES

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Some fatty acids (FA) influence the differentiation of adipocytes and may be ligands for peroxisome proliferator-activated receptor γ (PPARγ). To determine the effect of FA on transcripts for PPARγ, adipocyte differentiation determination factor 1 (ADD1), adipocyte fatty acid binding protein (aP2), and lipoprotein lipase (LPL), porcine stromal vascular cells were differentiated to adipocytes in vitro with or without FA. Addition of 50 to 300 mM oleic acid (C18:1), linoleic acid (C18:2), conjugated linoleic acid (CLA), linolenic acid (C18:3), and arachidonic acid (C20:4) for 1 day significantly increased adipocyte differentiation. Docosahexaenoic acid (C22:6) had no effect on adipocyte differentiation. The mRNA concentrations for PPARγ, and LPL were increased by C18:1, C18:2, and C20:4, but not by C18:3 and C22:6. All the unsaturated FA tested significantly increased the aP2 mRNA concentration (two- to seven-fold). Regardless of the effect on differentiation, C20:4, C22:6, and CLA decreased ADD1 mRNA. These results indicate selected FA increase porcine adipocyte differentiation. Also, the regulation of the expression of ADD1, PPARγ, and aP2 by FA may be through different mechanisms. (Supported, in part, by USDA-NRI grant # 970-3641.)

362.8

EFFECTS OF ZINC DEFICIENCY AND FOOD DEPRIVATION ON γ-GLUTAMYLCYSTEINE SYNTHETASE EXPRESSION.

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We have previously shown that zinc (Zn) deficient male rats have high levels of oxidative damage. Given that increased concentrations of reactive oxygen species (ROS) can lead to changes in gene expression, we hypothesized that Zn deficiency would induce the transcription of ROS sensitive genes. γ-glutamylcysteine synthetase (γ-GCS) is the first enzyme in the synthesis of the tripeptide, glutathione, and it's expression can increase under conditions of oxidative stress. To test whether the expression of the heavy subunit of γ-GCS (γ-GCS-HS) is increased in Zn deficient rats, male rats were fed a Zn deficient (ZnD) (0.5 μg Zn/g) or a control (CON) (25 μg Zn/g) diet, ad libitum, for 2-3 weeks. A pair fed group (PF) was also included. Kidney γ-GCS-HS mRNA was assessed via Northern blotting analysis. Rats fed the Zn deficient diet began decreasing their food intake after 2-3 days, and thereafter consumed the diet in a 3-4 day cyclic pattern. ZnD and PF males had low levels of γ-GCS-HS mRNA compared to CON males. Given that the expression of γ-GCS-HS was lower in both the ZnD and PF groups, this outcome suggests that caloric reduction contributes to the regulation of γ-GCS-HS expression in the ZnD rats. Further investigation is needed to ascertain if the decrease in γ-GCS-HS mRNA is a consequence of Zn deficiency per se and/or inanition.

362.9

EFFECT OF DIETARY ZINC DEFICIENY ON HEPATIC CYTOCHROME P450 EXPRESSION IN RATS.

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To investigate the effects of dietary zinc deficiency on hepatic cytochrome P450 (CYP) enzyme expression, male weaning Wistar rats (n=6 or 7 per group) were randomly assigned to one of the following groups: zinc adequate (31mg/kg diet), marginally zinc-deficient (3mg/kg diet), pair-fed control for the marginally zinc-deficient rats, severe zinc-deficient (1mg/kg diet), or pair-fed control for the severe zinc-deficient rats. All rats were killed at 63 days of age. Marginal zinc deficiency decreased CYP2C11-mediated testosterone 2α- and 16α-hydroxylase activities by $\sim$40% when compared to the corresponding pair-fed control rats. By comparison, severe zinc deficiency reduced each of these activities by $\sim$60%. The decrease in CYP2C11 activity was accompanied by a similar reduction in CYP2C11 protein and mRNA levels. Neither marginal nor severe zinc deficiency affected CYP3A-mediated testosterone 6β-hydroxylase activity, but relative CYP3A2 mRNA level was decreased by 49% in severely deficient rats. CYP2A1-mediated testosterone 7α-hydroxylase activity was increased (2.4-folds) only in severely deficient rats, but relative CYP2A1, protein and mRNA levels were not affected. Serum testosterone concentration was decreased by 67% in the marginally deficient group and it was below the detection limit ($<$ 0.2 ng/ml) in the severely deficient rats. Overall, these results indicate that hepatic expression of CYP2C11 and CYP3A2 can be modulated by dietary zinc deficiency and the effects may be a consequence of the reduced serum testosterone levels.

362.10

Establishment of plasmid transfection method for studying gene expression in human intestinal Caco-2 cells

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Caco-2 cell line has been used as a model of human intestinal cells. Plasmid transfection of Caco-2 cells provides a mean to evaluate the effects of dietary compounds on gene expression in intestinal cells. Using pSV-β-galactosidase(β-gal) plasmid, different transfection conditions were examined in this study. Transfection efficiency was quantified by visualizataion of X-gal staining and colorimetric assay. Comparing between cells seeded at different densities(1-40,000 cells/cm^2 and grown for different durations(1-6 days) prior to the transfection, cells that were seeded at 40,000 cells/cm^2 for 1 day led to the highest transfection efficiency. Transfection efficiency was evaluated at 48- or 72-hours after the plasmid incorporation and found to be similar. Several transfecting reagents were compared. Calcium phosphate method (DNA prepared by 7-minute precipatation) coupled to 5-minute 10% glycerol shock in the dose-dependent increases in X-gal staining and color formation. Plasmid concentration of 2.5μg/ml medium (compared to 0.5, 1, 3.75, 5 μg/ml medium) provided an optimal efficiency of 3% as judged by staining. Replacing glycerol shock with DMSO shock led to extensive cell toxicity. Lipofectamine 2000 reagent(GIBCO-BRL) using OPTI-MEM as the transfecting medium gave comparable results as the $CaPO_4$-glycerol protocol wherease DEAE-dextran method, Effectene and Superfect yielded almost non-detectable level of transfection. We are using the $CaPO_4$-glycerol method for studying nutrient regulation of gene expression in Caco-2 cells.